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PATENT SPECIFICATION

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(19)

(54) METHOD FOR DETERMINING BOD AND APPARATUS FOR USE THEREIN

(71) We, AJINOMOTO CO., INC., a corporation organised under the laws of Japan, of 5—8, Kyobashi 1 Chome, Chuo-Ku, Tokyo 104 Japan, formerly of No. 6, 1-chome, Kyobashi, Chuo-Ku, Tokyo, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a method for rapid, simplified and quantitative determination of biochemical oxygen demand, using an oxygen-sensitive electrode together with fixed microorganisms.

Biochemical oxygen demand (hereinafter referred to as "BOD") is the quantity of dissolved oxygen to be consumed by microorganisms in the course of assimilation of organic compounds in waste water.

One of the most common methods for determining the BOD of waste water is the five day BOD test prescribed by Japanese Industrial Standard Committee, Testing Methods for Industrial Waste Water, JIS KO102, 33, (1974). This method has been employed as a standard pollution monitoring tool since 1936. This method. however, requires a five day incubation period and demands skill in the determination. Therefore, all of the waste water to be discarded has to be stored till the result is obtained. Additionally, the standard method is inconvenient in that it is carried out batchwise and non-automatically.

According to the present invention there is provided a method of determining the biochemical oxygen demand of an aqueous liquid containing organic material, which method comprises measuring the rate of current decrease of an oxygen-sensitive electrode in the solution, which decrease is caused by the consumption by fixed microorganisms of oxygen dissolved in the solution during the assimilation by the fixed microorganisms of the organic material in the solution; and calculating the biochemical oxygen demand from the rate of current decrease by making use of the relationship between the biochemical oxygen demand and the rate of current decrease.

The method of the present invention can be divided into two types of method, depending upon the spacing between the fixed microorganisms and the oxygen sensitive electrode. One of the methods is referred to herein as a "contact-type" method. In this method, the fixed microorganisms are in direct or close contact with the oxygen-sensitive electrode. The fixed microorganisms and the oxygen-sensitive electrode together form a microbial electrode. Therefore, this method is also referred to herein as a "microbial electrode" method. The other method is referred to herein as a "separate-type" method. In this method, the fixed microorganisms are separate from the oxygen-sensitive electrode in the BOD determination system.

Fixed microorganisms employed according to the present invention are those which are immobilized in a polymer matrix as an immobilized microorganism, or enclosed between a diaphragm membrane of an oxygen-sensitive electrode and a dialysis membrane covering the diaphragm of the oxygen-sensitive electrode.

The BOD of waste water can be determined in a short time, for example 5 to 20 minutes, according to the present invention. The response time in the BOD determination depends upon the thickness of the membrane, the quantity of fixed microorgansims, the pH and the temperature. It is preferable, especially in the microbial membrane method, that the fixed microorganisms be used in an amount sufficient to assimilat all of the organic material diffusing into the membrane, i.e. that the diffusion of organic material be the rat -determining step.

Microorganisms employed according to the present invention are those which are capable of consuming oxygen dissolved in solution in the course of assimilating organic material. Examples of suitable microorganisms are bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa*, mould such as *Aspergillus niger* and *Rhyzopus hormoscense* and actinomycetes such as *Streptomyces griseus*. Mixed microorganisms containing many species and separated from soils or active sludges are more preferably used. These mixed microorganisms are referred to herein as microorganisms. The microorganisms may be cultured under aerobic condition at a temperature in the range from 20 to 35°C for 10 to 30 hours in a conventional culture medium containing a carbon source, a nitrogen source, a minor nutrient element and inorganic ions. The microbial cells may then be harvested by centrifuging the cultured broth, washed with water and stored at a low temperature. An active sludge itself may be employed as microorganisms to be enclosed.

The oxygen-sensitive electrode employed according to the present invention can be of any conventional type.

In the following description, reference will be made, by way of example, to the accompanying drawings.

Figure 1 shows a microbial electrode comprising a membrane 1 in which microorganisms are immobilized, a diaphragm membrane 2 or an oxygen-sensitive electrode, a platinum cathode 3, a lead anode 4 and an alkaline electrolyte 5. Membrane 2 is in direct contact with the platinum cathode 3, and membranes 1 and 2 are tightly secured in place by rubber rings 6 and 6'.

Figure 2 shows another microbial electrode formed by smearing intact wet cells of microorganisms on a diaphragm 3 and covering the microbial layer 2 thus formed with a dialysis membrane 1 such as a regenerated cellulose membrane to keep the microorganisms in place. The electrode further consists of a platinum cathode 4, an aluminium anode 5, an electrolyte 6 and rubber rings 7 and 7'. In the electrode shown in Figure 2, the microorganisms may be held between membranes 1 and 3 in a solid carrier, such as a piece of filter paper, containing a definite quantity of intact microbial cells. This method is preferable since the amount of intact cells used may be easily controlled and the microbial electrodes thus obtained have uniform characteristics. The dialysis membrane employed may be a conventional one having microscopic holes through which organic material in waste water can pass but through which the enclosed microorganisms cannot pass.

A schematic diagram of the contact-type method of the invention is illustrated in Figure 3. In Figure 3, there is shown a microbial electrode 1, a flow cell 2, a magnetic stirrer 3 and a recorder 5. The determination system illustrated in Figure 3 is used for determining BOD continuously. A sample solution whose BOD is from 10 to 399 ppm is put into a flow cell 2, saturated with dissolved oxygen and stirred by the magnetic stirrer. The temperature of the sample solution is maintained at 20°C during the determination. The microbial electrode 1 is dipped into the sample solution and the current of the electrode is visually recorded on the recorder 5. The current of the electrode decreases markedly with time till it reaches a steady state, as illustrated in Figure 4. The decrease in current is caused by an uptake of dissolved oxygen by the fixed microorganisms of the microbial electrode in the course of assimilating organic material from the sample solution. The rate of the current decrease depends upon the BOD. The decrease in current in steady state is proportional to the BOD, as is shown in Figure 5. Therefore, the BOD can be calculated from the rate of current decrease, or decrease in current in a steady state, by virtue of the linear relationship between the rate of current decrease and the BOD.

The steady state attained in this method may be attributed to the fact that, in a steady state, the quantity of dissolved oxygen consumed by the fixed microorganisms is equal to that diffusing into the membrane from the sample solution.

The "separate-type" method, illustrated in Figure 10, is similar to "contact-type" method except that the fixed microorganisms 3 are attached not to the oxygen-sensitive electrode but to the bottom or side wall of a flow cell 5. The absorption of atmospheric oxygen by the sample solution 2 is prevented by a rubber stopper 4. In this system, the BOD determination is carried out in the same manner as described in respect of the "contact-type" method. Thus, when the oxygen-sensitive electrode 1 is dipped into the sample solution, the current of the electrode decreases linearly with time but, unlike the "contact-type" method, a steady state is not attained. In this case the current decrease for a definite period, for example 5 to 30 minutes is determined. The rate of current decrease, i.e. the current decrease for a definite period, is proportional to the BOD of the sample solution, as is illustrated in Figure 11.

In order to know the preferred conditions under which the BOD is determined by the present invention, an experiment was

carried out by the "separate type" method with immobilized microorganisms in accordance with the method described in Example 3 below. Figure 11 shows the relationship obtained in this experiment between the BOD and the current decrease, the number of abcissa indicating the BOD determined by the five day BOD test. Figure 14 shows the effect of the quantity of immobilized microorganisms on the rate of current decrease, Figure 15 shows the effect of temperature on the rate of current decrease, and Figure 16 shows the effect of pH on the rate of current decrease. The experiment indicates that the BOD determination by the present invention is preferably carried out at a temperature of from 15 to 40°C and a pH of from 6.5 to 7.5, and that the BOD is preferably in the range of from 10 to 350 ppm.

It should be noted that the BOD can be determined continuously at regular intervals by the present invention. Thus, the system illustrated in Figure 3 further comprises a flow meter 4 for aeration, quantitative flow pumps 6 and 7, a control timer 8, a carrier liquid reservoir 9 and a vessel for the solution 10 whose BOD is to be determined. A phosphate buffer solution (as carrier liquid) saturated with dissolved oxygen is continuously fed into flow cell 2 by pump 7 during the determination. The current of the electrode is recorded ("base current"). A sample solution is injected into the flow cell 2 by pump 7 for a time of 5 to 20 minutes and the peak of current proportional to BOD of the sample solution is recorded. After the "base current" has returned to its original level, another sample solution is injected into the flow cell. The figure of the peak to be recorded is almost symmetrical when the time of sample injection is short (5—10 minutes), but in the "contact type" method, as illustrated in Figure 7, a steady state current is obtained when the time of sample injection is somewhat long (15—20 minutes).

The time of sample injection should be decided by considering whether the organic material in the waste water is easily assimilated or not. Provided that a sample is injected for 6 minutes, the overall time for determination would be 20 minutes.

The method of the present invention is markedly increased in stability and the response time is reduced by using fixed microorganisms domesticated with a phosphate. When an oxygen-sensitive electrode is dipped in deionized water saturated with dissolved oxygen, together with fixed microorganisms, the current of the electrode attains at a constant level, but, when a phosphate is added to the water, the current of the electrode decreases almost to zero even though the water contains no organic compounds to be assimilated. This current decrease is caused by the endogenous respiration of fixed microorganisms. Accordingly, the BOD of waste water containing phosphates cannot be determined unless fixed microorganisms which are domesticated with a phosphate, and accordingly do not result in endogeneous respiration when phosphate is added, are used. For the purpose of domesticating fixed microorganisms, it is preferable to use a phosphate buffer solution having a concentration of from 10^{-1} to 10^{-3}M during the determination. A phosphate affects fixed microorganisms at a stronger than 10^{-1} M concentration and does not play its role below 10^{-3}M. In addition to a stabilizing effect, a phosphate reduces response time in the determination since the phosphate accelates the assimilation of organic compounds by the fixed microorganisms.

The method of the present invention is not effected by injurious heavy metal ions such as Hg^{2+}, Cd^{2+}, Pb^{2+}, Fe^{3+} and Cu^{2+}, as shown in Table 1.

TABLE 1
Influence of Heavy Metal Ions

Ion	Current (uA)
None	25
Cu^{2+}	37
Hg^{2+}	25
Cd^{2+}	25
Fe^{2+}, Fe^{3+}	25
Pb^{2+}	25

The results in Table 1 were obtained by the contact type method, using a collagen-microbial membrane, at 30°C and using 10% of a standard solution containing 1 m M metal chloride. As shown in Table 1, a slight increase in the current was observed only in the case of Cu^{2+}; the currents in the other cases remained unchanged.

As regards the influence of NaCl, the oxygen-sensitive electrode is stable below a 0.2 M concentration of NaCl but a current increase is observed above 0.2M.

There is no essential difference between the method using immobilized microorganisms and the method using enclosed microorganisms, but the latter is preferable since it is easier to enclose the microorganisms than to fix them. In addition, the latter method continues to be sensitive without any change, except a slight drift in the base line, for at least 10 days in repeated and continuous determinations. On the other hand, in the former m thod, especially when a microbial-collagen membrane is used, a change in the "base current" som times occurs in continuous determination, whereby it is not possible to

use the former method for continuous and repeated determination for such a long period as the latter method.

Fixed microorganisms, once prepared, can usually be preserved without any loss of activity for at least 3 months. For example, when a microbial electrode with microbial-collagen membranes is preserved in a phosphate buffer solution (0.1 M, pH 7.0) containing 0.25 moles of glucose at 3°C for 3 months, no change in activity is observed.

As described above with reference to the drawings an extremely rapid, simplified and continuous BOD determination can be achieved with highly reliable reproducibility and precision. This new method for BOD determination will be useful for protecting humans against environmental organic pollution.

The invention will now be illustrated by the following Examples.

Example 1

A 10 ml soil extract prepared by the method prescribed by JIS KO102-1974 was cultured under aerobic conditions at 30°C for 24 hours in a 80 ml culture medium (pH 6.0) containing 10% of glucose, 1% of peptone, 1% of beef extract and tap water.

The microbial cells were harvested by centrifuging the cultured broth at 5°C and washed twice with phosphate buffer solution (0.1 M, pH 7.0). A 1.0 g portion of the wet cells was suspended in 100 g of 1% collagen-fiblile suspension (pH 4.0) in accordance with the method reported by Karube et al; Ind. Eng. Chem. Pro. Res. Develop., 10—160 (1971). Then, the suspension was casted on polytetrafluoroethylene board and dried at 20°C.

The collagen membrane obtained was immersed in aqueous 0.1% glutalaldehyde solution for 1 min. at 20°C.

The microbial electrode with microbial-collagen membrane as illustrated in Figure 1 was built by covering an oxygen-sensitive electrode (Model A oxygen-sensitive electrode manufactured by Ishikawa Seisakusho, Tokyo) with square microbial-collagen membrane 1 (2.5 cm + 2.5 cm) which was fitted tightly to the electrode with a rubber ring 6 and 6'.

A standard BOD solution prescribed by JIS and containing 150 mg glucose and 150 mg glutamic acid was prepared. Then, the solution was diluted with tap water to prepare three diluted standard solution containing 6, 16 and 22 ppm of the organic compounds as BOD. The microbial electrode obtained was inserted in 80 ml diluted standard solution stirred magnetically and saturated with dissolved oxygen as illustrated in Figure 3, and the current of the electrode was measured at 30°C which was visually recorded on the recorder. The time course of the current recorded was shown in Figure 4. In Figure 4, A, B and C are the currents of 6 ppm, 16 ppm and 22 ppm standard solutions, respectively.

On the other hand, BOD of the three standard solutions were also determined by the five day BOD test.

As shown in Figure 5, the decrease of current in steady state is apparently proportional to the BOD, as shown as solid line in the Figure.

BOD of the diluted waste water from the Japanese government alchohol factory, Inage, Chiba, was determined at various dilutions by the five day BOD test and the current decrease in steady state was determined in the same manner mentioned above. The current decrease and BOD obtained were plotted (open circles) in Figure 5. As shown in Figure 5, the current decrease vs BOD plots of the waste water are located near by the standard curve obtained from the standard solutions.

BOD of the same waste water was determined in the same manner 3 times per day for 10 days to confirm the stability of the microbial electrode.

The currents of the electrode measured were almost constant in the determination as shown in Figure 6.

Example 2

A 40 ml active sludge returned from an active sludge equipment of a food manufacturing factory was cultured under aerobic condition in 500 ml culture medium containing 1% of glucose, 1% of peptone, 1% of yeast extract and 0.5% of NaCl (pH 6.0) at 30°C for 10 hours.

The wet cells of microorganisms cultured were harvested by centrifuging the cultured broth at 5°C.

A microbial electrode as illustrated in Figure 2 was built by smearing the wet cells on a polytetrafluoroethylene diaphragm of an oxygen-sensitive electrode 3 (ST type oxygen-sensitive electrode manufactured by Oriential Denki) and covering wet cells layer 2 with a regenerated cellulose dialysis membrane 1 and continuous BOD determination system as illustrated in Figure 3 was built.

In this system, a phosphate buffer solution 9 (0.05 M, pH 7.0) was fed at a flow rate of 4 ml/min. to flow cell 2 (30 mm × 28 mm, its volume is 8 ml) by constant flow pump 6, and the current of the microbial electrode 1 was recorded as base line.

After the base line had come to constant level, the standard solution prepared by the JIS method were injected for 30 minutes at a flow rate 0.03 ml/min. by constant flow pump 7.

Then the second and third standard solution were injected in the same manner after the base line had returned to the original base line level. On the other hand, the BOD of the standard solutions was determined by the five day BOD test. The result obtained was illustrated in Figures 7 and 8. In Figure 7, A, C, E, G and I are the currents of the microbial electrode of the phosphate buffer solution and B, D, F and H are the current in steady state of 9, 18, 27 and 36 ppm of the standard BOD solutions, respectively, and the current of the standard BOD solution in steady state is, as shown in Fig. 8, directly proportional to the BOD.

A waste water of the food manufacturing factory was diluted with 0.05 M of phosphate buffer solution (pH 7.0) to a given concentration and the current of the microbial electrode prepared in the same manner as described above was determined. On the other hand, BOD of the same waste water was also determined by the five day BOD test.

The current decrease and BOD determined were plotted in Figure 8 (open circles). As shown in Figure 8, the current decrease vs BOD plots of the waste water are located near by the standard curve of the standard solution.

The current of the microbial electrode of the standard 36 ppm BOD solution and the phosphate buffer solution was determined continuously at regular time intervals.

The currents of the electrode in steady state measured were plotted as shown in Figure 9, in which A is that obtained in the phosphate buffer solution and B is that of standard 36 ppm BOD solution.

As shown in Figure 9, some drift was observed but in practice, the drift may be easily rectified with a standard BOD solution.

Example 3

Microorganism isolated from soil according to the method described in JIS, were cultured under aerobic conditions at 30°C for 24 hours in a medium (pH 7.0) containing 1% glucose, 1% peptone, 1% beef extract, and tap water. Then the microbial cells were harvested by centrifuging the cultured broth at 5°C, at 8000 × g, and washed twice with phosphate buffer (0.1 M, pH 7.0). A 0.3 g portion of the wet intact cells and 0.1 g mixture of two monomers (90% acrylamide and 10% N, N'-methylenebis-acrylamide) were suspended in 1 ml deionized water in a glass cell 5 (diameter, 4.7 cm, height, 5 cm). The polymerization reaction was initiated by addition of 0.25 ml of 100 g/l dimethyl aminopropionitrile and 12.5 mg potassium persulfate and was allowed to proceed anaerobically for 30 min. at 30°C.

The glass cell on the bottom of which the intact cells were immobilized by the polymerization reaction was directly used for this experiment, and BOD of the standard solution was determined in the BOD determining system as illustrated in Figure 10.

A 60 ml diluted standard solution prepared by the method described in Example 1 was placed in glass cell 5. Then the sample solution was saturated with dissolved oxygen and incubated at 30°C before the measurement. The oxygen-sensitive electrode 1 (Model T oxygen-sensitive electrode manufactured by Ishikawa Seisakusho, Tokyo) was inserted in the sample solution through rubber stopper 4 and air was completely excluded from cell 5 and the sample solution was stirred magnetically while the determination.

The current of the electrode was visually recorded and the rate of current decrease was determined 30 minutes after insertion of the electrode.

A linear relationship as shown in Figure 11 was obtained between the rate of the current decrease and the BOD determined by the five day BOD test.

In the same manner, the rate of the current decrease of the waste water from an alcohol factory were determined. The current decrease and BOD of the waste water is plotted (open circles) in Figure 11. As shown in Figure 11, the rate of current decrease vs BOD plots of the waste water are located near to the standard curve obtained from the standard solution.

Example 4

The influences of phosphate on the BOD determination of the present invention was examined by the following method. The microbial electrode built by the same manner described in Example 2 was dipped in distilled water and the current of the electrode was measured, as shown as A in Figure 12. Then the electrode was dipped in the standard solution (18 ppm BOD) containing no phosphate and the current decrease was recorded, as shown as B.

After determining of the current of distilled water again which is shown as C, the current decrease of the same sample solution but containing 0.16 M phosphate (used as buffer solution, pH 7.0) was recorded. The current decreased rapidly near to zero level, as shown as D. Successively, the current of distilled water was recorded, but it did not return to A level in a short time as shown as E.

This phenomenon is based on an endogenous respiration of the enclosed microorganisms of the electrode because the same current decrease was caused when the electrode was dipped in an only 0.16M

phosphate buffer solution containing no organic compounds.

On the other hand, two kinds of standard solution of which BOD were all 18 ppm were prepared, one of them contained no phosphate and the other contained 0.16M phosphate.

The current of the electrode was recorded in the same manner described above except that the 0.05M phosphate buffer solution was used instead of distilled water.

As shown in Figure 13, the current decreases of the two standard solutions were almost equal, and the response time to reach steady state is reduced in the presence of phosphate.

WHAT WE CLAIM IS:—

1. A method of determining the biochemical oxygen demand of an aqueous liquid containing organic material, which method comprises measuring the rate of current decrease of an oxygen-sensitive electrode in the solution, which decrease is caused by the consumption, by fixed microorganisms, of oxygen dissolved in the solution during the assimilation by the fixed microorganisms of the organic material in the solution; and calculating the biochemical oxygen demand from the rate of current decrease by making use of the relationship between the biochemical oxygen demand and the rate of current decrease.

2. A method according to claim 1, wherein the fixed microorganisms are spacially separated from the oxygen sensitive electrode.

3. A method according to claim 1, wherein the oxygen-sensitive electrode is in direct or close contact with the fixed microorganisms, whereby the oxygen-sensitive electrode and the fixed microorganisms together form a microbial electrode.

4. A method according to claim 3 wherein the microbial electrode comprises the oxygen-sensitive electrode and a membrane containing immobilized microorganisms.

5. A method according to claim 4, wherein microbial membrane is a microbial-collagen membrane.

6. A method according to claim 3, wherein the microbial electrode comprises the oxygen-sensitive electrode and a dialysis membrane between which the microorganisms are held.

7. A method according to any of claims 1 to 6, wherein the microorganisms are microoganisms which have been domesticated with a phosphate.

8. A method according to any claims 1 to 11, wherein the microorganisms are microorganisms which have been domesticated with a phosphate buffer solution having a concentration of from 10^{-1} to 10^{-3}M.

9. A method of determining biochemical oxygen demand, substantially as described in any one of the foregoing Examples.

10. A method of determining biochemical oxygen demand, substantially as hereinbefore described with reference to Figure 3 or 10 of the accompanying drawings.

11. A microbial electrode substantially as hereinbefore described with reference to Figure 1 or 2 of the accompanying drawings.

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Sheet 1

Fig. 1.

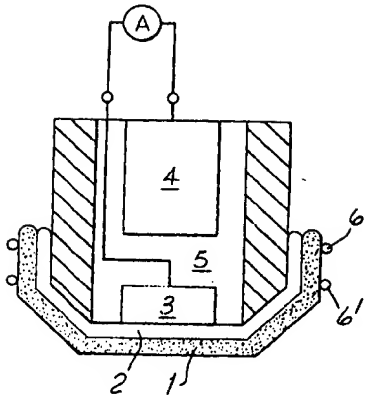

Fig. 2.

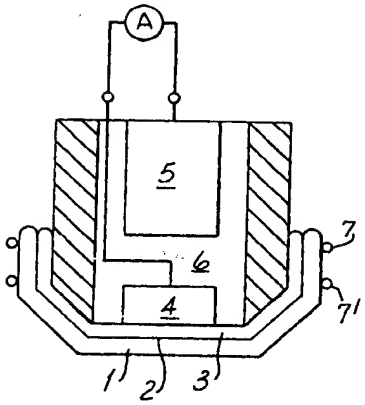

Fig. 3.

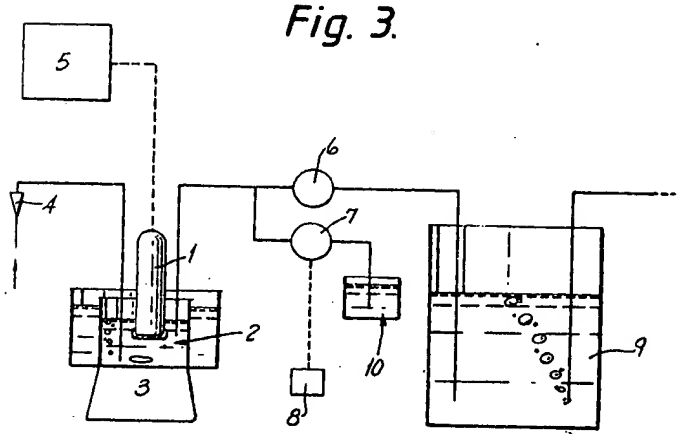

Fig. 4.

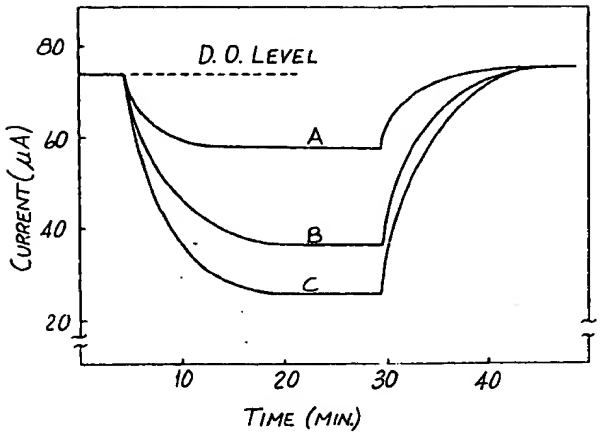

Fig. 5.

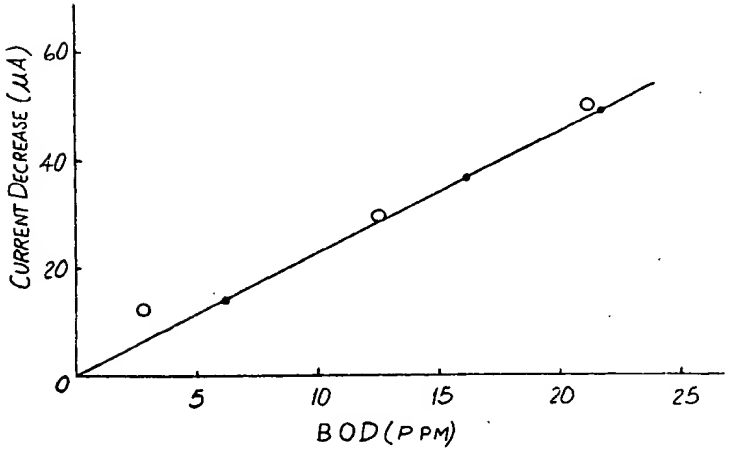

Fig. 6.

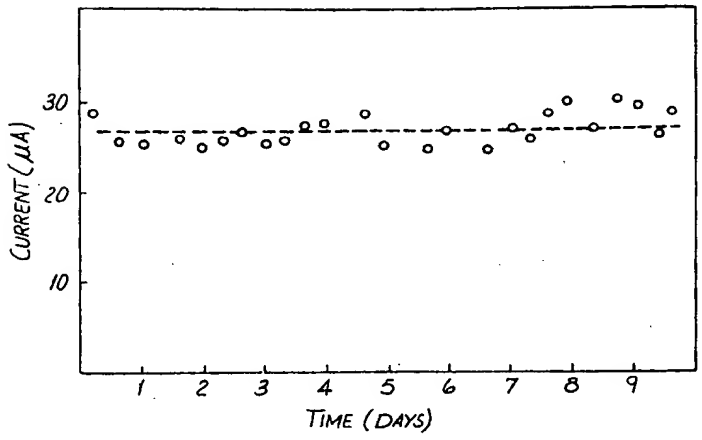

Fig. 7.

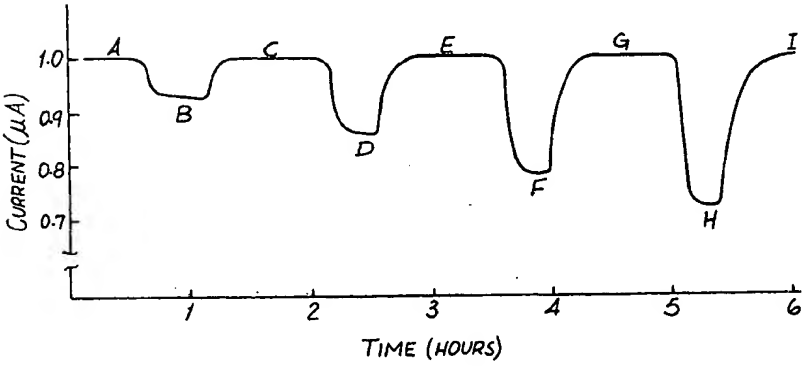

Fig. 8.

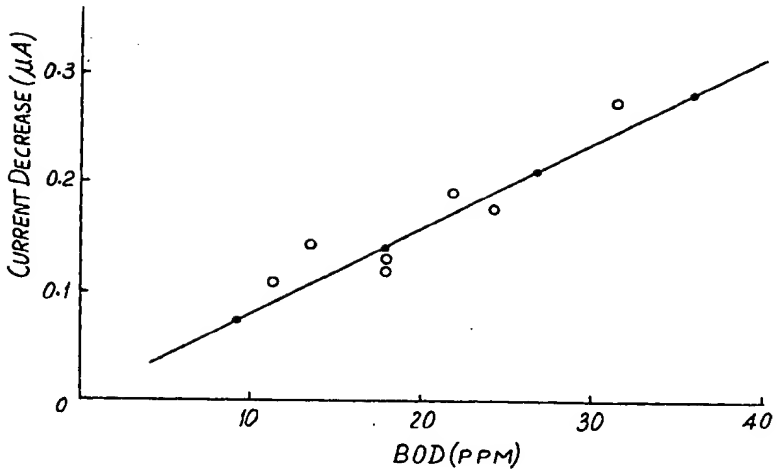

Fig. 9.

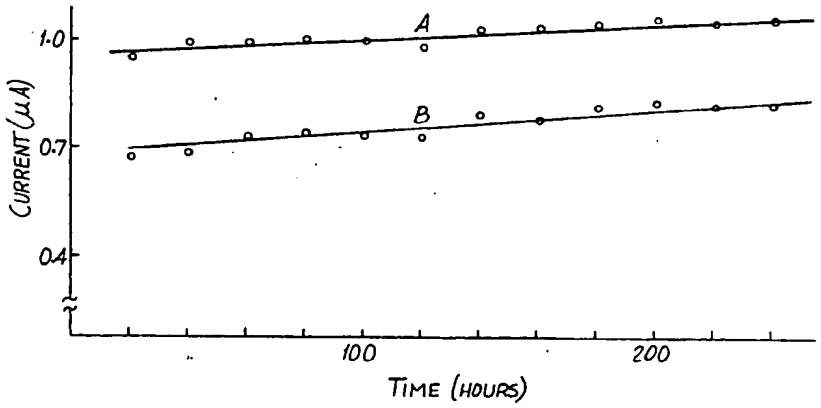

Fig. 10.

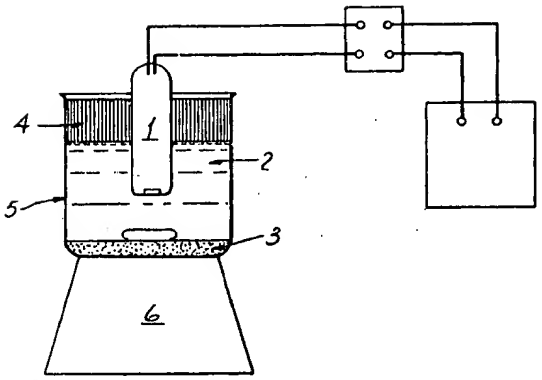

Fig. 11.

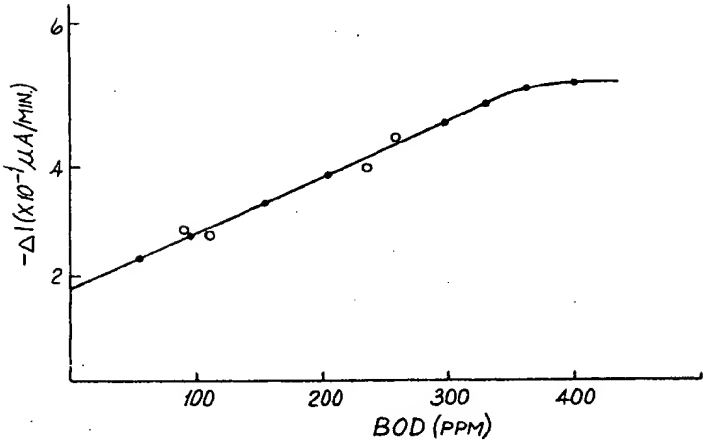

Fig. 12.

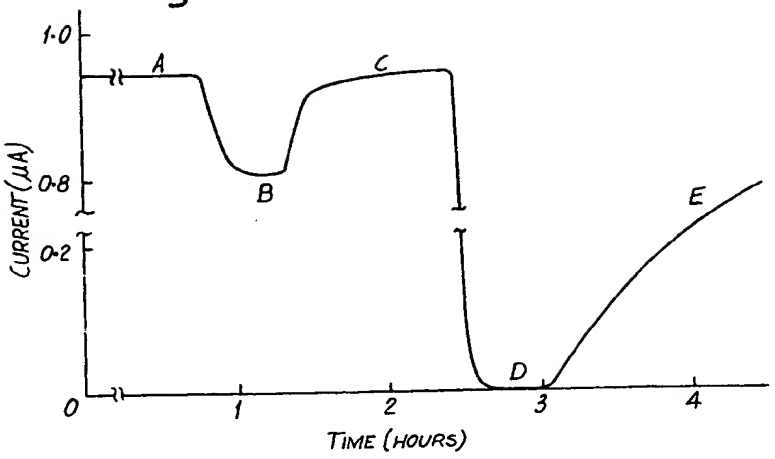

Fig. 13.

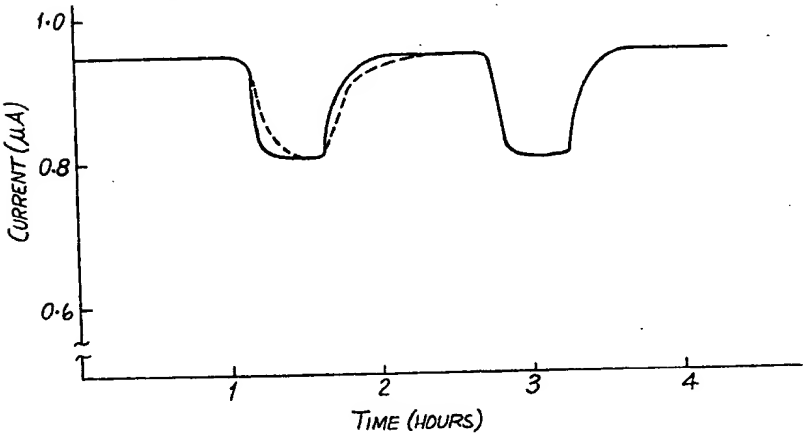

Fig. 14.

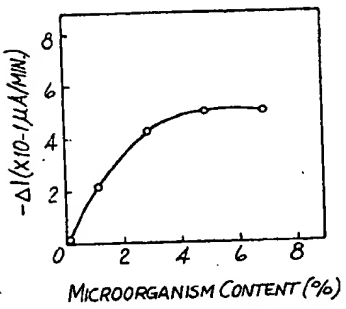

Fig. 15.

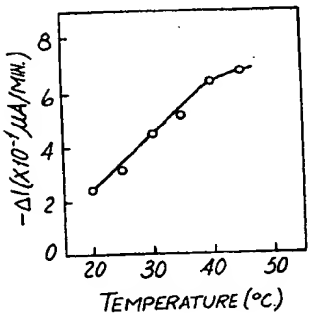

Fig. 16.